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(54) Title: METHOD OF DETECTING SCHIZOPHRENIA BY MEASURE OF GLUTATHIONE LEVEL IN THE BRAIN

(57) Abstract: The invention relates to a method for detecting schizophrenia, which comprises measuring glutathione level in the brain using proton magnetic resonance spectroscopy.

Method of detecting schizophrenia by measure of glutathione level in the brain

The present invention relates to a method for detecting schizophrenia, which comprises measuring glutathione (GSH) level in the brain and optionally in the cerbrospinal fluid (CSF) of patients.

It is known from K.Q.Do et. al., J. Neurochem. 65, 2652-2662 (1995), that γ-glutamylglutamine (γ-Glu-Gln) concentration is decreased by about 16% in the CSF of drug-naive patients with schizophrenic disorders. A significant decrease of taurine and a significant increase of isoleucine have also been reported. Based on this knowledge, a discriminant analysis using the independent variables aspartate, glutamate, γ-Glu-Gln, isoleucine and taurine performed on a population of schizophrenics and controls was reported, allowing correct classification of 82.9% of the subjects.

In view of the above, there is a need to provide a method which will detect schizophrenia with an improved rate of success. There is also a need to provide a non-invasive method suitable for detecting schizophrenia in a large population of patients.

It has now surprisingly been found that the level of GSH is significantly decreased by about 27% in the CSF of schizophrenic patients.

More importantly, it has surprisingly been found, using a non-invasive proton magnetic resonance spectroscopy (^{1}H-MRS) method developed for detection of GSH with high selectivity, that the GSH level is similarly decreased by about 50% in the medical prefrontal cortex of schizophrenia patients.

Based on the first mentioned finding, a new discriminant analysis using the above-mentioned variables and including additionally GSH was performed. On a population of schizophrenics and controls, said method allowed correct classification for 87.4% of the subjects, with a specificity which raised up to 96.2% of correctly classified patients and a sensitivity of 66.7% of correctly classified controls.

Based on the second mentioned finding, a non-invasive ^{1}H-MRS analysis of GSH in the medial prefrontal cortex was performed.

According to the above, a non-invasive method for detecting schizophrenia comprising ^{1}H-MRS analysis of GSH in the brain is provided. In a preferred embodiment, patients who in said analysis exhibit a significant decrease of GSH compared to controls are subjected to an analysis of GSH in the CSF. These two analyses taken together constitute a suitable method of detecting schizophrenics in large populations of subjects, whereby (a) lumbar puncture is only effected on a limited group of selected patients and (b) a high degree of correctly diagnosed patients is achieved.

The above-mentioned decrease of GSH level in the CSF of schizophrenic patients has been evidenced in a group of 26 drug naive (21) or drug free (4 for 1 year and 1 for 8 years) schizophrenic patients in whom long-term changes secondary to previous antipsychotic treatment could be excluded. The control group consisted of 14 subjects. The CSF sampling procedure was as described in Do. et al. (see above). The samples were analyzed by HPLC following derivatization with N-9-fluorenylmethyl-chloroformate (FMOC-Cl). The FMOC derivative of GSH was then identified by micro HPLC continuous flow fast atom bombardment mass spectrometry (FAB-MS). GSH was significantly decreased by 27% ($p< 0.05$) in the patients compared to controls. No effects of age and gender could be ascertained for the CSF concentration of GSH. Thus GSH decrease is unlikely to be related to pathologies of degenerative disorders of later age.

The CSF analysis was performed as follows:

The sample were thawed slowly on ice and deproteinated by ultrafiltration. Aliquots of samples containing norvaline were treated with 10 mM dithiotreitol to convert all GSH to its reduced from. They were derivatized at pH 8 with FMOC-Cl (15.5 mM in acetone) for 1 min. The excess of reagent was extracted with n-pentane and aliquots of the water phase were injected onto the HPLC column in duplicate. Analytical column (125x4mm), packed with Lichrospher 100, RP-18, 5 µm, 100 Ä (Merck), was used. The compounds were eluted at 40°C with a linear gradient of 0-65% mobile phase B [0.1 % trifluoroacetic acid (TFA) in acetonitrile /methanol/water (70:20:10% v/v)] in mobile phase A [0.1 % TFA in water] during 80 min at a flow rate of 0.8 ml/min. Fluoroscence was monitored at 315 nm (emission) and

260 nm (excitation). The FMOC-derivative of authentic GSH eluted at the retention time of 75 min. The quantitation was based on peak area measurements. To show that the derivatized CSF component termed P75 was indeed the FMOC-derivative of GSH, as suggested by its retention time, the eluent containing P75 was collected, and subjected to micro HPLC-continuous flow FAB-MS.

The above-mentioned decrease of GSH level in the brain of schizophrenic patients has been evidenced using a new ^{1}H-MRS method that allows detection of GSH with a high selectivity. In conventional in vivo ^{1}H-MR spectra GSH is not visible due to its rather low concentration, its complicated spectral pattern and spectral overlapping with other resonance lines. From the three amino acid components of GSH, cysteine was the most suitable for the identification of GSH by means of ^{1}H-MRS. For the selective detection of cysteine, a double quantum coherence filter technique based on coherence pathway filtering with static field gradients in combination with spatial selection of a single volume was used. 14 male patients participated in this study. The control group consisted of 14 age-matched subjects. The volume of interest (VOI) that comprised 17.4 ml (24 x 22x 33 mm) was placed mid-sagittally in the prefrontal cortex, an established site of dysfunction in schizophrenia [Andreasen et al. ID: 7581 (1992)]. In the control group, a mean ratio GSH signal/water signal of 6.12 ($\pm$ 2.82)x10^{-5} was found, compared to 2.95 ($\pm$ 1.48)x10^{-5} in the patients. The GSH level in the prefrontal cortex was thus decreased by 52% in the patients compared to controls ($p = 0.0012$; Mann-Whitney Test).

The ^{1}H-MR Spectroscopy was performed as follows:

From the 3 amino acids components of GSH, cysteine was found to be the most suitable for the identification of GSH by means of NMR spectroscopy. Cysteine forms a strongly coupled ABX spin system. In the ^{1}H-NMR sepctrum of cysteine, two separated multiplets centered in the 4.4 ppm and 2.95 ppm regions may be detected. The focus was on the 2.95 ppm resonance of GSH as it is located in a spectrally less crowded region. Other resonances found in this frequency region which potentially contribute to the observed in vivo spectrum are creatine (singlet at 3.03 ppm), aspartate (multiplet at 2.82 ppm) and GABA (triplet at 3.01 ppm). For the selective detection of cysteine, a double quantum coherence filter technique was used, based on coherence pathway filtering with static field gradients in combination with spatial selection of a single volume by means of the PRESS

technique [Bottomley et al. Proc. N.Y. Acad. Sci. 81 6856-6860 (1998)]. In addition the radio frequency read pulse was made frequency selective for the sake of a higher signal yield. To secure optimal and reproducible phase correlation between the radio frequency pulses, a calibration procedure was developed. The sequence was implemented on a Philips Gyroscan ACS NT (Philips Medical Systems, Best, The Netherlands) 1.5 Tesla whole body scanner. The double quantum filter technique provides excellent background discrimination between the cysteine compound of GSH and the uncoupled creatine spins. A non-negligible fraction of signal from aspartate leaks through the filter. In vitro experiments showed that the spectral resolution is sufficient to separate GSH and aspartate signal on the basis of the differences in their chemical shifts. A minor contribution to the observed signal originates from GABA, which is negligible for the poor yield in combination with the low concentration of GABA.

Quantification was accomplished by using tissue water content as an internal standard. Due to the complex spin dynamics of the cysteine spin system the signal ratio GSH / Water does not directly reflect the ratio [GSH]/[Water]. Therefore no exact absolute concentrations of GSH in brain tissue may be derived from the data. Estimations gave an average GSH concentration in the range of 2-4 mM for the control group, in keeping with biochemical measurements.

In addition to descriptive statistics (mean ± SD) an analysis of covariance (ANCOVA) was performed, with age and sex as the covariates. In case of significant main group effects, group-by-group comparisons were calculated using Student's t tests (modified least significant difference tests to control for the increased type I error rate). In addition, linear canonical discriminant analysis (minimizing Wilk's lambda) was performed using the known set of independent variables (Asp, Glu, γ-Glu-Gln, Ile, Tau).

In accordance with the above, the present invention provides a method for detecting schizophrenia, which comprises measuring GSH level in the brain using ^{1}H-MRS.

Preferably GSH level is measured in medial prefrontal cortex.

Preferably the method additionally comprises subsequent determination of GSH level in the CSF.

Preferably in addition to GSH level, levels of one or more further variables e.g. selected from aspartate, glutamate, γ-Glu-Gln, isoleucine and taurine are also determined in the CSF.

CLAIMS:

1. A method for detecting schizophrenia, which comprises measuring glutathione level in the brain using proton magnetic resonance spectroscopy.

2. A method according to claim 1, wherein glutathione level is measured in medial prefrontal cortex.

3. A method according to claim 1, which additionally comprises subsequent determination of glutathione level in the cerebrospinal fluid.

4. A method according to claim 3, which additionally comprises determination of one or more further variables.

5. A method according to claim 3, which additionally comprises determination of one or more further variables selected from aspartate, glutamate, γ-glutamylglutamate, isoleucine and taurine.

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(54) Title: METHOD OF DETECTING SCHIZOPHRENIA BY MEASURE OF GLUTATHIONE LEVEL IN THE BRAIN

(57) Abstract: The invention relates to a method for detecting schizophrenia, which comprises measuring glutathione level in the brain using proton magnetic resonance spectroscopy.

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
INSPEC, BIOSIS, EPO-Internal, WPI Data, PAJ, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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20 November 2000	21/12/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Muñoz, M

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Interi nal Application No
PCT/EP 00/05129

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 00/05129

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ›nal Application No
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